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Identification of fibronectin binding sites in dermatopontin and their biological function

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ABSTRACT

Background: Dermatopontin (DP) is a 22 kDa acidic extracellular matrix (ECM) protein that plays a critical role in both ECM structure and wound healing. Previously, we demonstrated that DP interacts with fibronectin (Fn) and promotes formation of insoluble Fn fibrils that are called activated Fn (Kato et al., J Biol Chem 2011;286:14861–9).
Objective: Details of the interaction between DP and Fn are investigated to further examine the biological functions of DP.
Methods: Interactive sites between Fn and DP were examined by a solid-phase assay using Fn, DP, and their respective recombinant proteins and synthetic peptides. The effect of the DP peptides on insoluble Fn fibril formation was examined by both electrophoresis and electron microscopy.
Results: A binding site in DP for Fn was identified as the DP-4 (PHGQVVVAVRS) peptide, and the major binding site in Fn for DP was the 14th type III repeat (III_{14}) domain. Further, the major DP binding site in the III_{14} domain was located around the B- and C-strands and their connecting loop region. A synthetic cyclic peptide mimicking the Fn loop structure enhanced DP binding activity. The DP-4 peptide induced Fn polymerization but the morphology was different from that of Fn fibrils formed by full length DP. The Fn fibrils with DP-4 enhanced integrin $\alpha5\beta1$-mediated cell adhesion and spreading.
Conclusion: Interactive sites between Fn and DP were identified. The DP-4 peptide activated Fn and enhanced cell adhesion activity. DP-4 has the potential to be used for therapeutic applications, such as a wound treatment.

1. Introduction

Dermatopontin (DP) is an acidic dermal protein that comprises approximately 50 mg/kg wet weight of the dermis [1–6]. DP interacts with a proteoglycan decorin and regulates collagen fibrillogenesis [7–9]. The importance of DP in forming the extracellular matrix (ECM) structure was highlighted by analysis of DP knockout mice, where the phenotype demonstrated Ehlers–Danlos syndrome, showing fragile and hyperelastic skin [10,11]. Then, it was reported that expression of DP is enhanced and sustained in the area of myocardial infarction, suggesting that DP expression is related to the wound healing process [12].

We previously demonstrated that DP is a potent cell adhesion molecule for the epidermal keratinocyte cell line, HaCaT [13]. We also identified the cell surface receptors for DP as $\alpha3\beta1$ integrin and syndecan-1. We further showed that the syndecan-1 binding site in DP is DP-4 (PHGQVVVAVRS) [13]. Recently, it was shown that expression of DP in cultured cardiac fibroblasts is enhanced by hypoxia, and that DP promotes adhesion and migration of fibroblasts [14]. Taken together, these reports suggest that DP may have multiple roles in wound healing.

Accordingly, we have found DP in a provisional matrix as well as in both the wound fluid and the serum [15]. The provisional matrix is an initial ECM, which is formed just after wounding, that serves as an early structure to support wound healing [16–18]. The

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provisional matrix is composed mainly of fibrin and fibronectin (Fn), and these components serve as an adhesive surface for both fibroblasts and endothelial cells [19,20]. We have found that DP enhances the interaction between Fn and fibrin, and that DP polymerizes soluble Fn resulting in insoluble Fn fibrils. The Fn fibrils are called activated Fn, and activated Fn enhanced fibroblast adhesion activity [15]. Recently, we reported that DP binds fibrin/fibrinogen and accelerates fibrin formation [21]. The fibrin fibrils formed in the presence of DP demonstrated enhanced endothelial cell adhesion. Thus, DP is a molecule that interacts with other matrix components and can modify their structures and biological activities.

Activated Fn promotes enhanced cell adhesion [22] and has anticancer activity [23–25]; hence activated Fn may have therapeutic uses. As mentioned above, DP can activate Fn, however, the amount of DP is limited. Therefore, instead of using DP for activating Fn, an active peptide derived from DP would be beneficial since it can be produced chemically and in a large amount. Therefore, we sought to identify the active DP peptide which participates in the interaction with Fn and in the formation of activated Fn.

2. Materials and methods

2.1. Materials

DP was purified from newborn calf dermis. Synthetic DP peptides and an anti-DP carboxyl-terminal peptide antibody were produced as reported previously and the sequences of the DP peptides are shown in our previous publication [7]. The sequences of deletion peptides are shown in Table 1. Human plasma Fn, an anti-Fn monoclonal antibody, 70-kDa thermolysin fragments, a recombinant anastellin, and a 120-kDa chymotryptic fragment of Fn were purchased from Sigma (St. Louis, MO). Retronectin, which is a fragment spanning between III_9 and an amino-terminal part of the V region of Fn lacking III_{11}, was purchased from TaKaRa Chemical (Tokyo, Japan). A carboxyl-terminal fragment, namely fragment 4, was purchased from R & D systems (Minneapolis, MN). Function-blocking anti-integrin subunit anti bodies (FB12 for α1, P1E6 for α2, ASC-1 for α3, P1D6 for α5, M9 for αv, NKI-GoH3 for α6, and 6S6 for β1) and bovine fibrinogen were purchased from Chemicon (Temecula, CA). Sulfo-NHS-LC-biotin, horseradish peroxidase (HRP)- conjugated streptavidin was purchased from Pierce (Rockford, IL). Two partial III_{14} peptides, III_{14}d and III_{14}e (shown in Fig. 3A), were produced and purchased from Hokkaido System Bioscience (Sapporo, Japan). Human dermal fibroblasts were

Table 1
Sequences of DP-4 related peptides.

DP-4	PHGQVVVAVRS
DP-4S	VRVHVPVQGSA
DP-4a	-HGQVVVAVRS
DP-4b	–GQVVVAVRS
DP-4c	——VVVAVRS
DP-4e	PHGQVVVAVR-
DP-4f	PHGQVVVAV–
DP-4g	PHGQVVVA—
DP-4h	PHGQVVV——

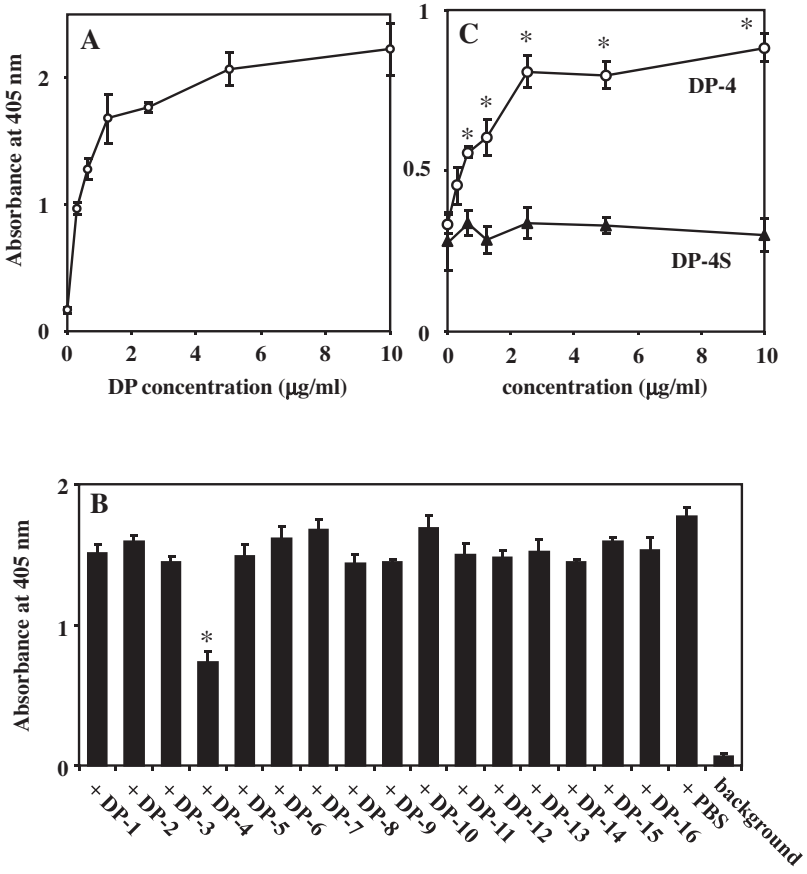

Fig. 1. Interaction between Fn and DP and their respective component peptides. (A) Interaction between Fn and DP. Fn was immobilized on 96-well plates and DP at the indicated concentrations was added. (B) Effect of DP peptides on the interaction between Fn and DP. Fn at 1 μg/ml was immobilized, and mixtures of DP at 2 μg/ml and synthetic DP peptides (100 μg/ml) were added. An asterisk indicates $p \leq 0.05$. (C) Interaction between Fn and DP-4. Immobilized Fn was incubated with either biotinylated DP-4 or DP-4S at the indicated concentrations. Asterisks indicate $p \leq 0.05$. In A–C, experiments were done in triplicate, and data are expressed as the averages ± S.D.

prepared according to the guidelines of the Ethical Council of Oita University.

2.2. Production of recombinant domains of Fn

The production of III_{13} and III_{14} was performed using a pCOLD I bacterial expression system (TaKaRa Bio, Tokyo, Japan) at 15 °C for 24 h using *Escherichia coli* BL-21 as a host, as reported previously [15]. For expression of partial peptides within III_{14}, the pCOLD-TF vector (TaKaRa Bio) was used. A sense primer 5′-GAATTCAATCCTGGCACGTGGCGGCTG-3′ and an antisense primer 5′-GGATCCGCCATTGATGCACCATCCAAC-3′ spanning between A_{1902}–I_{1930}, a sense primer 5′-GAATTCGACACCAGGGCGGGGCCGAGG-3′ and an antisense primer 5′-GGATCCACCGGCTACATCATCAAGTAT-3′ spanning between T_{1931}–V_{1955}, and a sense primer 5′-GAATTCTGTCTTTTTCCTTCCAATCAG-3′ and an antisense primer 5′-GGATCCACAGAGGCTACTATTACTGGC-3′ spanning between T_{1956}–T_{1991} were produced and purchased from Hokkaido System Science (Sapporo, Japan). All the antisense primers were designed to have a BamHI sequence, and the sense primers were designed to have an EcoRI sequence at their 5′-ends, respectively.

These peptides were expressed as fusion peptides connected to a transcription factor (TF), and were purified as described previously [15]. The final purified peptide solution was lyophilized and dissolved in PBS.

2.3. Biotinylation of DP-4 and DP-4S peptides

Because the amino-terminal amino acid of the DP-4 peptide is proline, a glycine was added to the amino-terminus. The glycine-DP-4 and DP-4S (control) peptides were labeled with sulfo-NHS-LC-biotin according to the manufacturer's instructions as reported previously [21]. The labeling was confirmed by Western blot probed with HRP-conjugated streptavidin. The biotinylated glycine-DP-4 peptide is called biotinylated DP-4 peptide in this study.

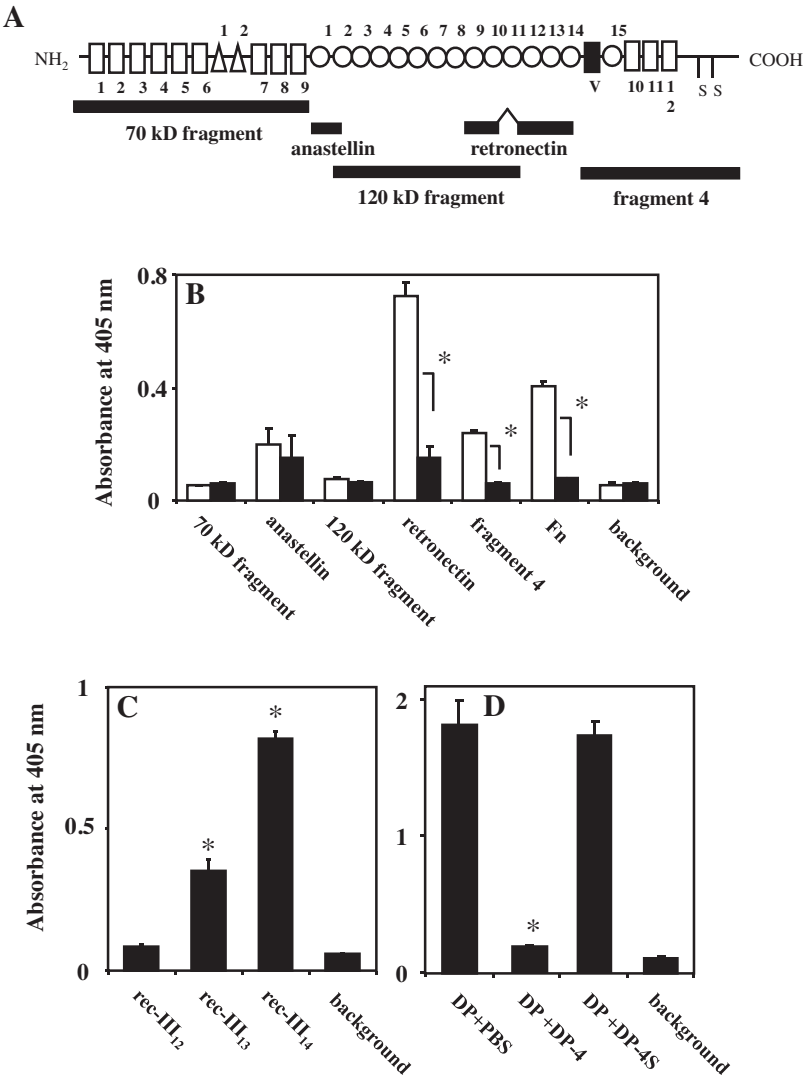

Fig. 2. Identification of DP-4 interaction sites in Fn. (A) Location of the Fn fragments. Schematic model of the Fn monomer molecule is shown on the top. Rectangles indicate type I repeats, triangles indicate type II repeats, circles indicate type III repeats, and a black rectangle indicates a V region. The numbers of each repeat are shown along the characters. The Fn fragments are expressed as black bars and are placed at their corresponding locations. (B) Screening of DP-4 interaction sites in Fn. Biotinylated DP-4 (white bar) or DP-4S (black bar) at 10 μg/ml was incubated with immobilized Fn fragments. (C) Interaction between DP-4 and recombinant Fn III domains. Recombinant Fn III domains expressed in *E. coli* were immobilized on plates and biotinylated DP-4 at 10 μg/ml was added. (D) Inhibition of the interaction between III_{14} and DP by DP-4. Fn III_{14} was immobilized, then DP at 2 μg/ml was added in the presence of either 100 μg/ml of DP-4 or DP-4S. In B-D, asterisks indicate $p \leq 0.05$ and experiments were done in triplicate, and data are expressed as the averages ± S.D.

2.4. Solid-phase assay for protein interactions

All procedures were carried out as described previously [8,15]. Briefly, proteins were immobilized overnight on 96-well plates and were blocked with 1% BSA. DP, biotinylated peptides, Fn, or the mixture were incubated overnight. The bound DP and Fn were probed with their corresponding antibodies, followed by HRP-conjugated secondary antibodies. Biotinylated peptides were probed by HRP-conjugated streptavidin. After color development with 2,2′-azido-bis (3-ethylbenzothiazoline-6-sulfonic acid), the absorbance at 405 nm was determined. The concentration of proteins and peptides for immobilization was 10 μg/ml, until otherwise mentioned.

2.5. Fibril-formation assay

Fn fibril formation was assessed according to a previous report [15]. DP peptides were added to 0.5 μM Fn solution and incubated for 16 h at room temperature. After incubation, the pellet and supernate were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a conventional 5% gel under reducing conditions. For control, scrambled peptide or adjacent peptides were mixed with Fn and were processed.

2.6. Electron microscopy

One μM Fn was incubated with 300 μM DP-4 peptide in PBS at room temperature for 8 h. The samples were placed on a poly-L-lysine-coated coverglass and were immobilized at room temperature for 10 h in a wet chamber. The samples were then treated with Karnovsky solution, washed with cacodylic acid, stained with 1% OsO4 and 1% tannic acid, dehydrated, dried, and coated with OsO4. All the observations and photographs were performed with a scanning electron microscope (S-4800, Hitachi, Tokyo, Japan) operated at an acceleration voltage of 15 kV.

2.7. Cell culture

Normal human dermal fibroblasts were cultured as a monolayer in 75-cm^2 bottles at 37 °C in a humidified 5% CO_2, 95% air atmosphere. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum.

2.8. Cell adhesion assay

The assays were done as previously described [13,15]. Briefly, Fn and DP peptides were mixed and incubated for 6 h. Then, the

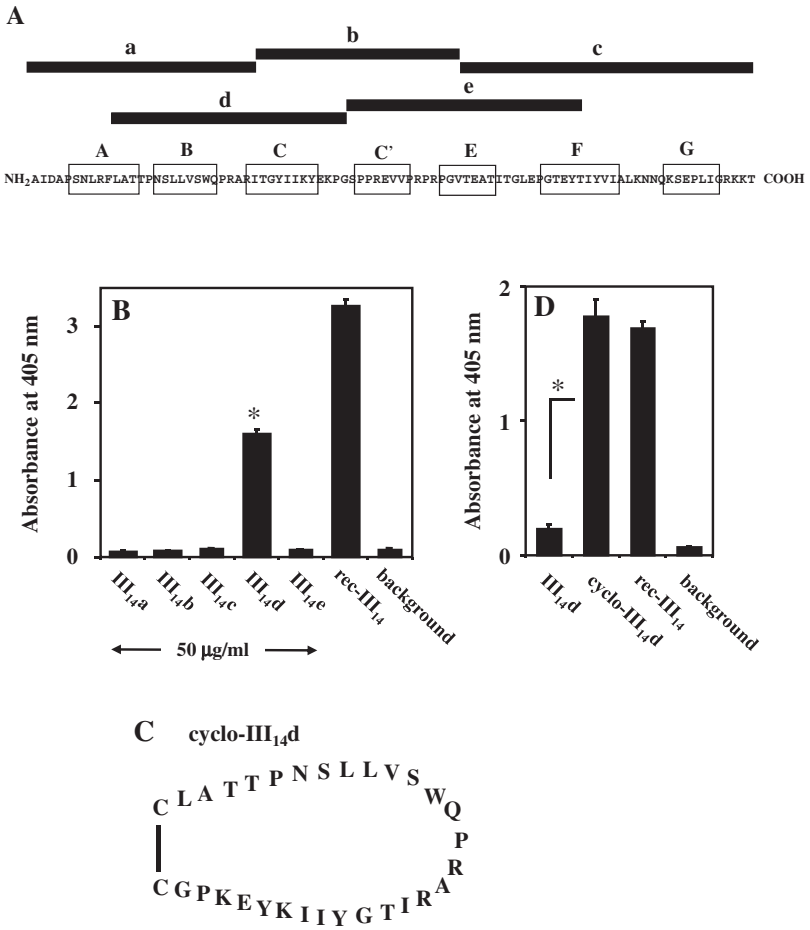

Fig. 3. Identification of DP binding sites in the Fn III_{14} domain. (A) Location of synthetic peptides in the III_{14} domain. Peptides are indicated by black bars and the amino acid sequence of III_{14} is shown at the bottom. Rectangles along the sequence indicate β sheets. (B) Screening of interaction with DP. The III_{14} partial peptides at 50 μg/ml or Full-length rec-III_{14} domain at 10 μg/ml were immobilized, and DP at 2 μg/ml was added. (C) Primary structure of the cyclo-III_{14}d peptide. (D) DP binding to III_{14}d and to cyclo-III_{14}d. Peptides and rec III_{14} domain at 10 μg/ml were immobilized, and DP at 2 μg/ml was added. In B and D, asterisks indicate $p \leq 0.05$. The experiments were done in triplicate, and data are expressed as the averages ± S.D.

mixtures were coated on 96-well plates. After blocking, fibroblasts were inoculated at 30,000 cells/100 μl/well in DMEM and were incubated at 37 °C for one hour. After fixation and staining of the attached cells, cell adhesion was quantified by measuring the absorbance at 595 nm.

2.9. Statistical analyses

Statistical assessments for protein interaction and cell adhesion were performed by a Student *t*-test and a *p* value under 0.05 was considered as significant.

3. Results

3.1. Identification of Fn binding sites on DP

DP bound to immobilized Fn in a dose-dependent manner (Fig. 1A), and the binding profile was identical to that in our previous report [15]. An inhibition study of the interaction between DP and Fn was performed using sixteen sequential peptides that covered the entire DP sequence (Fig. 1B). The DP-4 peptide significantly inhibited DP binding to Fn but the other peptides did not. The direct interaction between DP-4 and Fn was examined using biotinylated DP-4 and a scrambled control DP-4 peptide, DP-4S. The biotinylated DP-4 peptide interacted with Fn in a dose-dependent manner, while the biotinylated DP-4S peptide did not (Fig. 1C). The result indicates that the DP-4 sequence in the DP molecule is critical for interaction with Fn.

3.2. Identification of DP-4 interactive sites in Fn

Next, we screened DP-4 binding sites in the Fn molecule using commercially available Fn fragments (Fig. 2A). Retronectin and fragment 4 bound to DP-4 (Fig. 2B). The DP-4 peptide binding to retronectin was much stronger than that of fragment 4, suggesting that the major Fn binding site for DP-4 is contained within retronectin. A 120 kD fragment, which partially overlaps with retronectin, did not interact with DP-4, suggesting that the DP-4 binding site locates in a stretch between the III_{12} and III_{14} domains.

Next, we screened the DP-4 binding sites in the Fn $III_{12–14}$ domains using three recombinant proteins (rec-III_{12}, rec-III_{13}, and rec-III_{14}). DP-4 interacted with rec-III_{13} and rec-III_{14} but did not interact with rec-III_{12} (Fig. 2C). Additionally, the DP-4 binding affinity to rec-III_{14} was stronger than that to rec-III_{13}. These results suggest that the Fn major binding site for DP-4 is the Fn III_{14} domain. Further, the interaction between rec-III_{14} and DP was significantly inhibited by the DP-4 but not by a control peptide DP-4S (Fig. 2D). These results suggest that the DP-4 sequence is critical in the DP molecule for binding to the Fn III_{14} domain.

3.3. Identification of the major DP binding site in the Fn III_{14} domain

Further, we analyzed DP binding sites in the Fn III_{14} domain using five overlapping peptides that covered the entire domain (Fig. 3A). First, these peptides were immobilized, and DP binding was examined. When the peptides were immobilized using 10 μg/ml concentration, DP binding was not observed (data not shown). When they were immobilized using 50 μg/ml concentration, only the III_{14}d peptide significantly bound to DP (Fig. 3B). This result suggests that the III_{14}d peptide has potential to interact with DP and that some crucial feature of the III_{14}d peptide is required.

The III_{14} domain is composed of seven β-sheet strands based on crystal structure analysis [26]. The Fn III_{14}d peptide is located on the B- and C-strands in their connecting loop region. To mimic the loop structure, a cyclic peptide cyclo-III_{14}d was prepared by adding

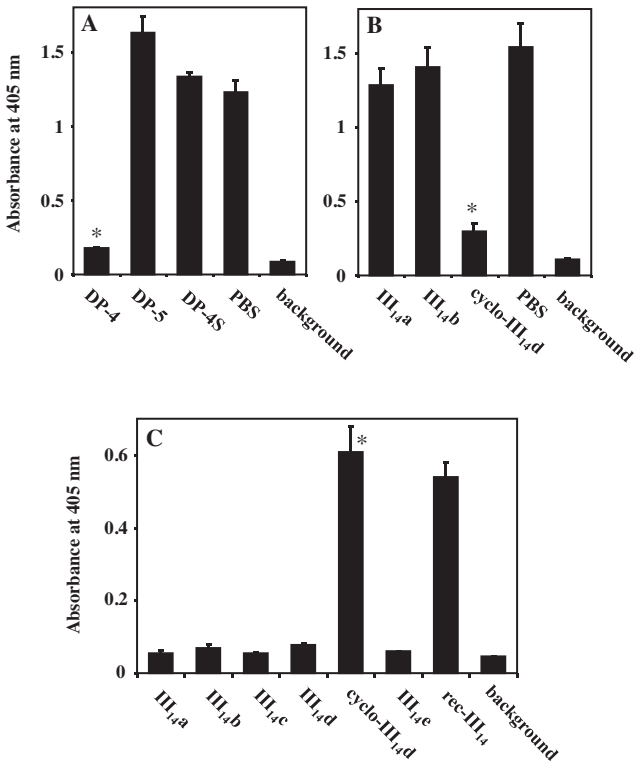

Fig. 4. Cyclo-III_{14}d peptide is the interaction site in Fn. (A) Inhibition of interaction by DP peptides. Cyclo-III_{14}d was immobilized, and the interaction with 2 μg/ml of DP was inhibited by DP peptides at 100 μg/ml. (B) Inhibition of interaction by truncated III_{14} peptides. Cyclo-III_{14}d was immobilized, and the interaction with 2 μg/ml of DP was inhibited by truncated III_{14} peptides at 100 μg/ml. (C) Direct interaction between DP-4 peptide and cyclo-III_{14}d. Truncated III_{14} peptides were immobilized, and biotinylated DP-4 at 10 μg/ml was added. In A–C, asterisks indicate $p < 0.05$. In A–C, experiments were done in triplicate, and data are expressed as the averages ± S.D.

two cysteines at the N- and C-termini of III_{14}d (Fig. 3C). DP bound to the cyclo-III_{14}d peptide immobilized with 10 μg/ml solution, whereas it did not bind to the non cyclized III_{14}d peptide that is immobilized at the same concentration (Fig. 3D). The DP binding affinity to the cyclo-III_{14}d peptide was equivalent to that of the Fn rec-III_{14}.

Next, we examined effect of the DP peptides on the interaction between cyclo-III_{14}d and DP. As expected, the interaction was inhibited by DP-4, whereas an overlapping peptide DP-5 and the scrambled peptide DP-4S had no activity (Fig. 4A). Additionally, the interaction was inhibited by cyclo-III_{14}d, but not by the III_{14}a and III_{14}b peptides (Fig. 4B). These findings suggest that the DP-4 and Fn III_{14}d sequences are specifically involved in the interaction between DP and Fn. Next, a direct interaction between DP-4 and cyclo-III_{14}d was examined. The DP-4 peptide clearly bound to the cyclo-III_{14}d peptide but did not bind to the other peptides (III_{14}a–e) (Fig. 4C). These results suggest that the loop structure of III_{14}d is important for binding to the DP-4 peptide.

3.4. DP-4 peptide induces formation of fibronectin fibrils

When Fn is activated, it forms insoluble fibrils [20]. Previously, DP was found to activate Fn and to enhance the production of Fn fibrils [15]. Here, we examined the effect of DP-4 on the formation of an insoluble Fn pellet. As shown in Fig. 5A, most of the Fn formed an insoluble pellet after incubation with DP-4, while the other peptides did not affect the formation of the insoluble pellet. DP-4 enhanced the formation of the Fn pellet in a dose-dependent manner (Fig. 5B). A study using the deletion peptides shown in

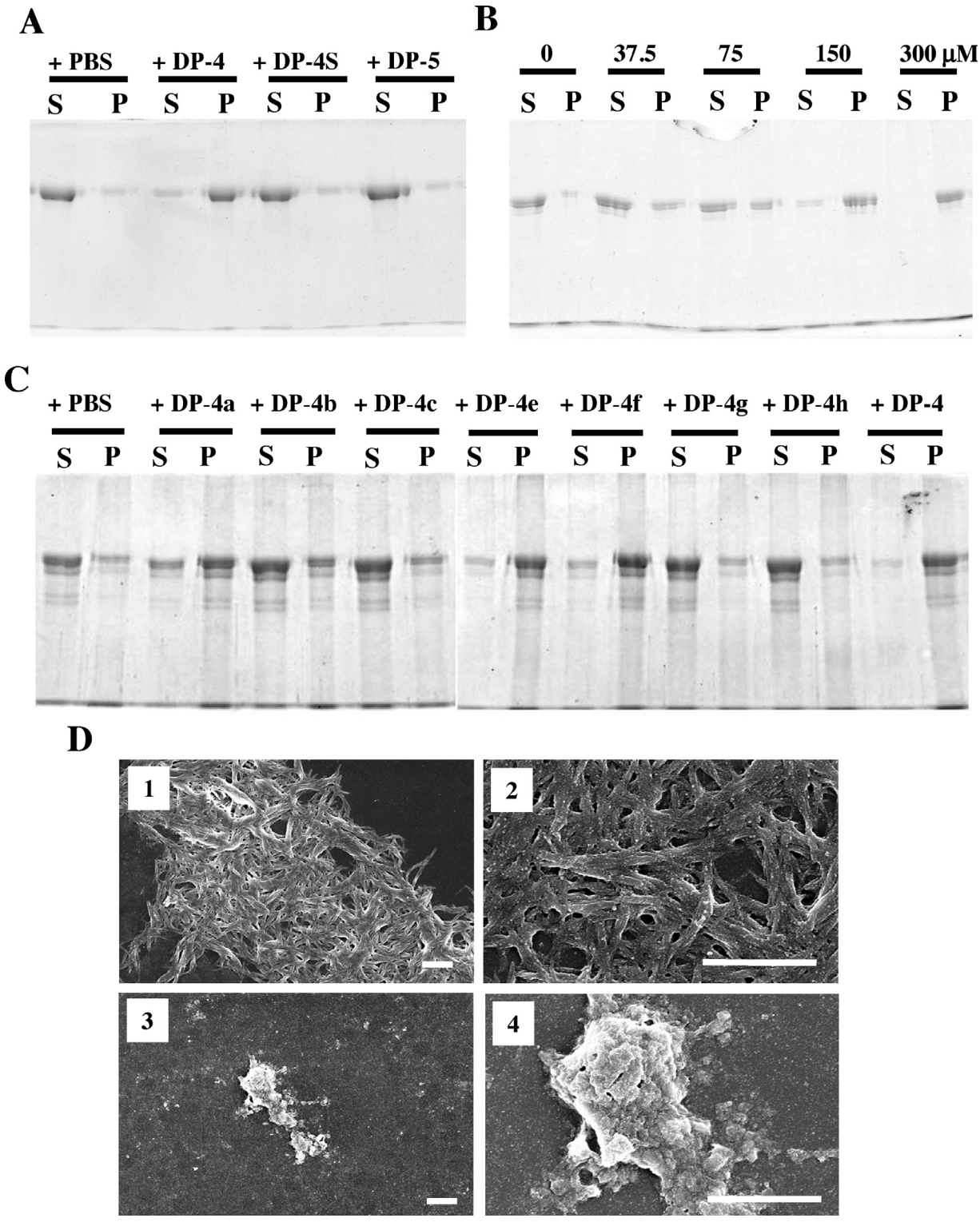

Fig. 5. Fn polymer formation with DP peptides. (A) Specificity of the DP-4 peptide for formation of Fn polymer. The Fn and DP peptides indicated on the top of the panel were incubated together, and then the supernate and pellet were separated and were analyzed by SDS-PAGE. Note that only DP-4 enhances the formation of a Fn pellet. The concentration of DP peptides was 300 μM. (B) Determination of minimal DP-4 concentration for forming Fn polymers. Fn was incubated with DP-4 at the concentrations indicated at the top of the panel, and the supernate and pellet were separated and analyzed as above. (C) Determination of minimal active sequence of DP-4 for formation of Fn polymers. Fn was incubated with the DP-4 deletion peptides indicated at the top of the panel, and was analyzed as above. The concentration of DP-4 deletion peptides was 300 μM. In A–C, S and P indicate supernate and pellet, respectively. (D) Morphology of Fn fibrils formed by DP-4. Fn was incubated with DP-4 and the suspension was placed on a glass coverslip and the sample was prepared for electron microscopy. Panels 1 and 2: Fn-DP-4 suspension. Panels 3 and 4: Fn only. Bars indicate 200 nm.

Table 1 demonstrated that a peptide lacking an amino terminal proline (DP-4a) retained the ability for Fn polymerization, whereas a peptide lacking two amino acids (DP-4b) lost the ability. In contrast, the peptide lacking two carboxyl terminal amino acids (DP-4f) enhanced Fn pellet formation but the peptide lacking three amino acids (DP-4g) did not (Fig. 5C). These results suggest that the minimal active sequence of the DP-4 peptide for Fn polymerization is HGQVVVAV.

To assess the formation of Fn fibrils, the insoluble pellet was examined by scanning electron microscopy (Fig. 5D). When Fn was incubated with DP-4, the Fn/DP-4 mixture formed numerous fibrils (Fig. 5D, panels 1 and 2). The Fn/DP-4 fibrils were 20–50 nm in diameter and shorter and straighter than those composed of Fn/DP as reported previously [15]. In contrast, Fn formed irregular aggregates in the absence of DP-4 and it did not form the fibrillar structures (Fig. 5D, panels 3 and 4). These results demonstrated that the DP-4 peptide promotes Fn fibril formation as well as DP.

3.5. Effect of peptides on cell adhesion to Fn fibrils

The effect of the DP-4 peptide on the cell adhesion activity of Fn fibrils was examined. The DP-4 peptide enhanced fibroblast adhesion to the Fn fibrils in a dose-dependent manner, and the

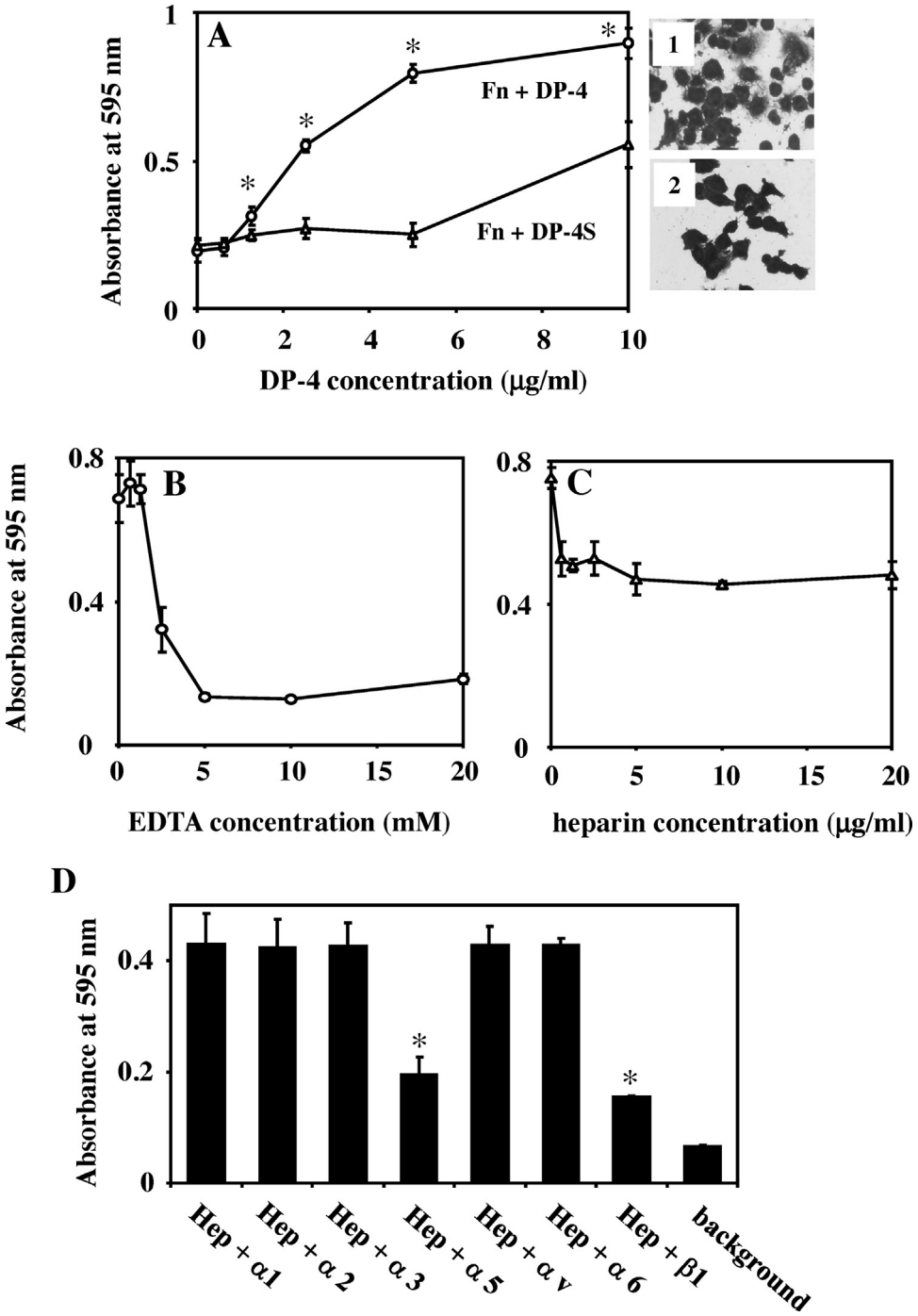

Fig. 6. Profiles of cell adhesion to Fn fibrils formed by DP-4. (A) Fibroblast adhesion to the Fn fibrils. Fn at 1 μg/ml was mixed with DP-4 at the indicated concentrations in PBS, and the mixtures were incubated for 6 h at room temperature. The mixtures were immobilized in wells, and cell adhesion was assayed. Circles indicate DP-4, and triangles indicate DP-4S. Panel 1: cells that adhered to fibrils made by Fn and DP-4 at 1 μg/ml and 5 μg/ml, respectively. Panel 2: cells that adhered to a mixture of the same concentrations of Fn and DP-4S. (B) and (C) Inhibition of cell adhesion by EDTA and heparin,. Fn at 1 μg/ml was incubated with 5 μg/ml of DP-4, and the mixture was immobilized on 96-well plates. Then, cell adhesion was assayed in the presence of either EDTA (B) or heparin (C) at the indicated concentrations. D: Inhibition of cell adhesion by mixtures of anti-integrin subunit antibodies and heparin. Cell adhesion was examined in the presence of anti-integrin subunit antibodies (10 μg/ml) and heparin (5 μg/ml). In A and D, asterisks indicate $p \leq 0.05$. In A–D, experiments were done in triplicate, and data are expressed as the averages ± S.D.

cell adhesion activity reached a plateau at a DP-4 concentration over 5 μg/ml. In contrast, a control peptide DP-4S enhanced cell adhesion to the Fn only at a higher concentration (10 μg/ml) (Fig. 6A). Further, the Fn fibrils formed with DP-4 promoted cell spreading but the fibrils formed with DP-4S did not promote cell spreading (Fig. 6A, panels 1 and 2). Next, we evaluated the effects of ethylenediaminetetraacetic acid (EDTA) and heparin on cell adhesion to the Fn fibrils formed with DP-4 (Fig. 6B and C). Cell adhesion on the Fn fibrils formed with DP-4 was inhibited completely by EDTA and partially inhibited by heparin. These data suggest that the Fn fibrils formed by DP-4 promote integrin- and heparan sulfate proteoglycan (HSPG)-mediated cell adhesion.

We next examined inhibitory effect of anti-integrin antibodies on the cell adhesion to the Fn fibrils formed with DP-4. Individual antibodies did not demonstrate inhibition of cell adhesion (data not shown). Therefore, we hypothesized that integrin and HSPG cooperate for cell adhesion. The effect of anti-integrin antibodies on cell adhesion was re-examined in the presence of heparin. In the presence of heparin, anti-integrin α5 and β1 antibodies significantly inhibited cell adhesion (Fig. 6D) whereas the other antibodies were inactive. These results demonstrate that the cell surface receptors of the Fn fibrils formed with DP-4 are α5β1 integrin and HSPG. Since the HSPG type receptor for DP-4 was identified as syndecan in our previous study [13], re-identification was not done in this study.

4. Discussion

In the present study, a functional binding site in DP for Fn was identified as the DP-4 peptide, and the major DP binding site in Fn molecule for DP was identified as the B- and C-strands and their connecting sequence in the III_{14} domain. The cyclic peptide mimicking the B- and C-strands connecting loop structure enhanced the DP binding activity, suggesting that the non linear structure is important for the interaction between Fn and DP. Further, the DP-4 peptide did not completely inhibit the interaction between Fn and DP, suggesting that there are additional binding sites in the both molecules.

The DP-4 peptide promoted Fn polymer formation and enhanced cell adhesion to Fn as well as DP. However, the DP-4 peptide promoted short and straight Fn fibrils, which differed from the longer winding Fn fibrils formed with DP in our previous report [15]. This result suggests that there are additional functional sites in the DP molecule for complete Fn fibril formation.

A model for the mechanism of Fn fibril formation suggests that inactive Fn assumes a folded conformation because of an intra-domain interaction between III_{2-3} and III_{12-14} [27]. When Fn is forming fibrils, the intramolecular interaction is broken, and new intermolecular interactions are created between I_{1-5} and III_{12-14}, and between I_{1-5} and III_{1-2} [28–32]. In our previous report, DP was shown to enhance Fn fibril formation by inhibiting the intramolecular interaction between III_{2-3} and III_{12-14} and by enhancing the intermolecular interaction between I_{1-5} and III_{12-14} [15]. In this study, we determined whether DP-4 also modifies those intra- and intermolecular interactions of Fn. Unexpectedly, DP-4 did not modify any of these interactions (data not shown). These results indicate that the mechanism of DP-4 peptide-induced Fn fibril formation is different from that by DP, and that the precise mechanism remains unclear.

Integrin α5β1 [33–35] is an important molecule that promotes Fn fibril formation. In contrast, only two non-integrin type molecules had been shown to enhance Fn fibril formation: anastellin, which is an 89-amino acid long fragment containing the carboxyl two-thirds of the Fn III_1 domain [22,36,37], and DP [15]. DP is the only authentic ECM protein that can promote the formation of Fn fibrils. Here, DP-4 was shown for the first time to promote the formation of Fn fibrils.

In this study, the DP-4 peptide enhanced the Fn fibril formation and cell adhesion. It has been reported that some non-adhesive proteins enhance cell adhesion to Fn, when Fn and the proteins were immobilized at the same time [38]. The mechanism of this phenomenon is not clear, but some Fn conformational change is likely occurring. We think that a similar effect of co-immobilization occurred in our assay, however, the DP-4 peptide clearly enhanced cell adhesion at a much lower concentration. These results indicate that the enhancement is sequence-specific to the DP-4 peptide. Cell surface receptors for the DP-4 peptide-induced Fn fibrils were α5β1 integrin and HSPGs, and these molecules are also the receptors for the Fn III_{10} domain and DP-4 peptide, respectively [39]. Therefore, it is likely that both III_{10} domain and DP-4 peptide are exposed on the surface of the Fn fibrils.

When Fn is activated by α5β1 integrin and anastellin, the Fn forms fibrils and cell adhesion and proliferation are accelerated [29,40–42]. Similar biological events seem to be taking place in the provisional matrix during wound healing. We have previously reported that DP is present in the provisional matrix and showed a possibility that DP can activate Fn before the cells migrate into the provisional matrix [15]. This finding suggests that when Fn and DP are mixed together as a matrix substrate, DP activates Fn and the resulting matrix induces cell adhesion and proliferation. Here, we showed that the DP-4 peptide regulates Fn fibril formation. Although the morphology of the fibrils was different from that formed via incubation of Fn and DP together, the peptide-induced fibrils enhanced cell adhesion. The synthetic DP-4 peptide has many advantages compared with the parental DP molecule to be used as a biomaterial and also has potential as a component of a novel artificial matrix that provides fibrillar Fn to wounds.

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